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TITLE: Dual Modulators of GABA-A and Alpha7 Nicotinic Receptors for Treating Autism

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14. ABSTRACT
Autism spectrum disorder (ASD) is a polygenic signaling disorder that may result, in part, from an imbalance in excitatory and inhibitory neurotransmission at the network level which strongly suggests that inhibitory neurotransmission plays a key role; perhaps as a result of deficits in g-aminobutyric acid-A receptor (GABAAR) mediated signaling. Therefore GABAARs may be a relevant therapeutic target for blocking or reversing the symptoms of ASD. Nicotinic cholinergic activity may supplement/enhance GABA efficacy on cognitive function. Significant cognitive deficits are associated with ASD and extensive preclinical studies/clinical proof-of-concept trials support a crucial role for a7 nicotinic acetylcholine (nACh) receptors in mediating learning and memory processes. The purpose of our proposal is to test the hypothesis that the simultaneous and selective positive allosteric modulation of GABAA & a7 nACh receptors will alleviate the core deficits and significant comorbidities of ASD. Our major findings are that selective positive allosteric modulation of ı 1-subunit containing GABAA receptor subtypes, by the test compound 2-261, impacts 1) aberrant behaviors reflected in sociability (social interaction) and 2) repetitive behavior (stereotypy) in the BTBR mouse model of ASD. These observations support the importance of the loss of inhibitory tone in these behavioral deficits and that the pharmacological enhancement this tone will help mitigate these deficits. Collectively, the observations made thus far support our working hypothesis and we will continue to test its validity in the coming year.

15. SUBJECT TERMS- Gamma-amino-butyric acid, inhibitory, excitatory, neurotransmission, autism, social interaction, stereotype behavior, autism spectrum disorder, nicotinic, receptor, mouse.

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1. **INTRODUCTION:** Autism spectrum disorder (ASD) is a disease of development characterized by a three core behavioral symptoms including difficulties in social interaction, verbal and nonverbal communication and repetitive/stereotypical behaviors. The Department of Health & Human Services states that the increasing prevalence of ASDs, currently estimated at 1 in 88 children, is a national health emergency. Yet there are no drugs for the treatment of these core deficits or associated difficulties such as epilepsy and anxiety. Consequently, ASD is a dire unmet medical need. The greatest challenge is to find a drug with a broad range of activity that will treat both the core symptoms and associated difficulties (i.e., epilepsy, anxiety, disrupted learning and memory). The objective of our project is to fulfill this profound need for drugs that can do exactly that by studying a new class of compounds that will simultaneously enhance the function of two neurotransmitters in the brain known as γ-aminobutyric acid (GABA) and acetylcholine (ACh). GABA acting through $GABA_A$ receptors ($GABA_ARs$) is responsible for reducing the activity of nerve cells in the brain that may be over-stimulated in ASD and thus contributes to the three core symptoms and the anxiety and epilepsy that sometimes occur in ASD. ACh acting through α7 nicotinic receptors (α7 nAChRs) control the activity of nerve cells in the brain that may be under-stimulated in ASD which may underlie the difficulties in learning and memory observed in ASD. This approach may provide the basis for the design of an innovative series of drugs that will represent the first attempt at treating the core and significant comorbidities of ASD with a single drug.

2. **KEYWORDS:**

Alpha7 nicotinic receptors (α7 nAChRs), allosteric modulator, anxiety, autism spectrum disorder, brain, childhood disorders, comorbidity, epilepsy, excitatory, γ-aminobutyric acid (GABA), GABA type A receptor ($GABA_AR$), inhibitory neurotransmitter, learning, memory, mouse, self-grooming, sociability, social interaction

3. OVERALL PROJECT SUMMARY:

The project summary covers the first year of the project and follows exactly the activities described under each task in the approved Statement of Work (SOW). The narratives of the activities related to each task appear in *italics*.

Task 1: Seek animal use approval through local Institutional Animal Care and Use Committee (IACUC) and Department of Defense (DoD) Animal Care and Use Review Office (ACURO).

Task 1 was accomplished in the middle of the month of October, 2013. All animal protocols for the proposed studies received approval from the IACUC at UC Irvine and the ACURO.

The initiation of a breeding colony to provide BTBR mice for the studies was started in November, 2013. Sufficient numbers of mice for studies specified in task 2 occurred in February, 2014 and took about one month longer than predicted in the approved SOW.

Task 2: (Specific Aim 1): Do single site positive allosteric modulators (PAMs) of α7 nACh or $GABA_A$ subtype (i.e., $\beta_{2/3}$-subunit containing) receptors correct any of the core ASD-related symptoms and comorbidities in the mouse models?

Task 2a: Synthesis of 2-261, AVL-3288 & GRN-529.

This task was accomplished in December, 2013. The task was accomplished one month later than predicted in the approved SOW because of the need to synthesize some of the starting materials that were commercially unavailable for the synthesis of the compounds of interest.

Task 2b: Training on behavioral paradigms using BTBR mice.

Training was completed in December, 2013 utilizing the BTBR mice from by the breeding colony. This task was completed one month later than anticipated due to the one month delay in breeding BTBR mice in sufficient numbers to satisfy demand as described in task 1.

Task 2c: Pharmacokinetics – confirm brain penetrability in BTBR mice.

The brain penetrability of the test compounds 2-261 and AVL-3288 were determined in BTBR mice and found to be brain penetrant and consistent with observations in other strains of mice that we have tested in the past. The plasma and brain levels of 2-261 following a 10 mg/kg i.p. dose is shown in table 1. The brain levels of 2-261 achieved at 10 mg/kg i.p. are well in excess of that required to activate the receptor (when based on the EC_{50} of 2-261 at the $GABA_A$ receptor i.e., saturating concentrations) as measured electrophysiologically. Comparable brain levels of AVL-3288 were also achieved after a 10 mg/kg i.p. dose (data not shown). This task was completed in February, 2014, about one month later than predicted in the SOW due to a one month delay in generating sufficient numbers of BTBR mice in the breeding colony.

Table 1. Plasma and brain levels of 2-261 at 30 and 60 minutes after a 10 mg/kg i.p. dose

Time (minutes) after i.p. administration	Plasma levels (μM)±SEM	Brain levels (μM)±SEM
30	1.5 ± 0.3	4.3 ± 1.0
60	0.6 ± 0.1	2.3 ± 0.4

SEM=standard error of the mean

Task 2d: Sociability – phenotype confirmation (BTBR strain).

The behavioral phenotype of the BTBR strain of mice was confirmed and found to be consistent with that reported in the literature (Silverman et al., 2012). The phenotype is shown in left panel of figure 1 where BTBR mice spend significantly more time with the novel object over the novel mouse (DBA/2) in this sociability paradigm. As found in published reports from other groups, we have also observed no statistically significant differences in time spent between the novel object and mouse but we have never observed a significantly greater spent with the novel mouse over the novel object. These studies were completed in March, 2014, one month later than anticipated due to the delay in generating sufficient numbers of BTBR mice in the breeding colony.

Task 2e: Sociability – phenotype confirmation (C57 strain).

The behavioral phenotype of the C57 strain of mice was confirmed and consistent with that reported in the literature (Silverman et al., 2012). The phenotype is shown in the right panel of figure 1 where C57 mice spend significantly more time with the novel mouse than the novel object in this sociability paradigm. We also observed sessions where no differences in sniffing times between the novel object versus novel mouse. However we have never observed cases where C57 mice show increased interaction with the novel object over the novel mouse which is consistent with literature reports. These studies were completed in March, 2014, one month later than anticipated due to the delay in generating sufficient numbers of BTBR mice in the breeding colony.

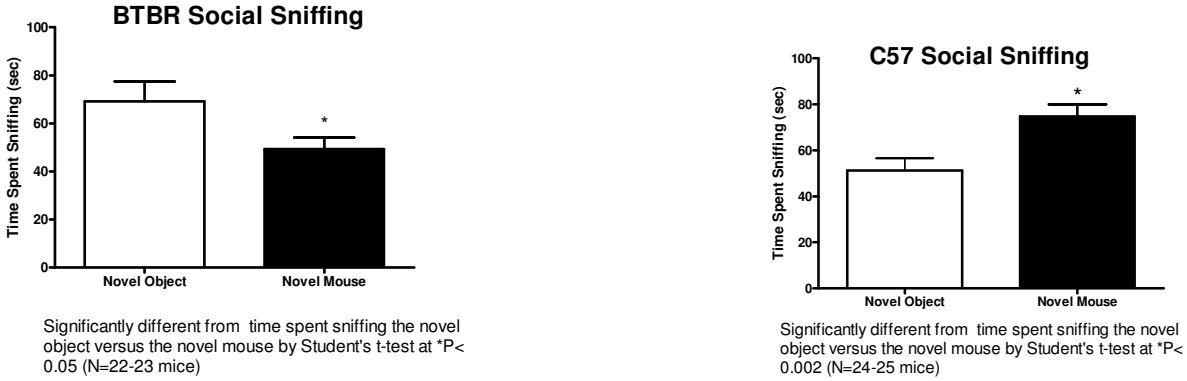

Figure 1: The sociability of BTBR (left panel) versus C57 (right panel) strains of mice in the 3 chamber test where mice were placed in a center chamber and allowed interact with a novel object (open bar) or a novel mouse (DBA/2 strain, filled bar) on either side of the center chamber for a test session of 10 minutes duration. Time spent in seconds (sec) sniffing the novel mouse or the novel object was recorded.

Task 2f & 2g: Sociability – drug testing (BTBR strain).

Three drugs, the positive control GRN-529, 2-261, AVL-3288 and vehicle were tested in the BTBR mice to determine their effects on sociability as measured by the difference in time they spend with a novel object versus a novel mouse (DBA/2) in the sociability paradigm. Figure 2 shows the effect of the positive control GRN-529 (3 mg/kg i.p.) on sociability in BTBR mice. Consistent with the effect of this compound reported in the literature (Silverman et al., 2012), it significantly alters the preference of the BTBR mice by reducing the time spent with the novel object while increasing time spent with the novel mouse. When the $GABA_A$ receptor subtype selective modulator 2-261 was tested in this paradigm, it showed an effect similar to GRN-529 where the behavioral phenotype was altered to reflect reduced time spent with the novel object at a dose of 1 mg/kg i.p. (figure 3). The effect was dose-dependent with no significant effect observed at a dose of 0.3 mg/kg i.p. (data not shown).

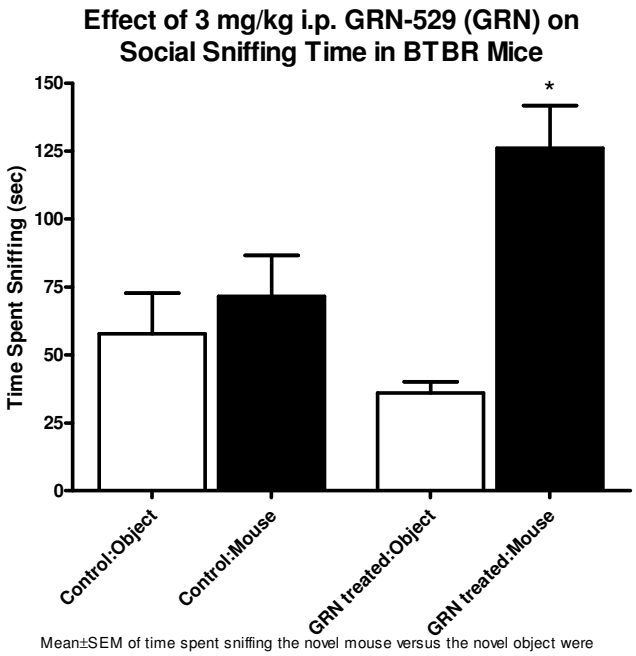

Figure 2: The effect of the positive control GRN-529 (GRN, 3 mg/kg i.p.) on sociability (3 chamber test) in BTBR mice was recorded as the time in seconds (sec) spent sniffing the novel mouse (Mouse) versus the novel object (Object). The effect of the vehicle (Control) is shown in the left pair of bars and the drug (GRN-529 treated) effect is depicted in the right pair of bars.

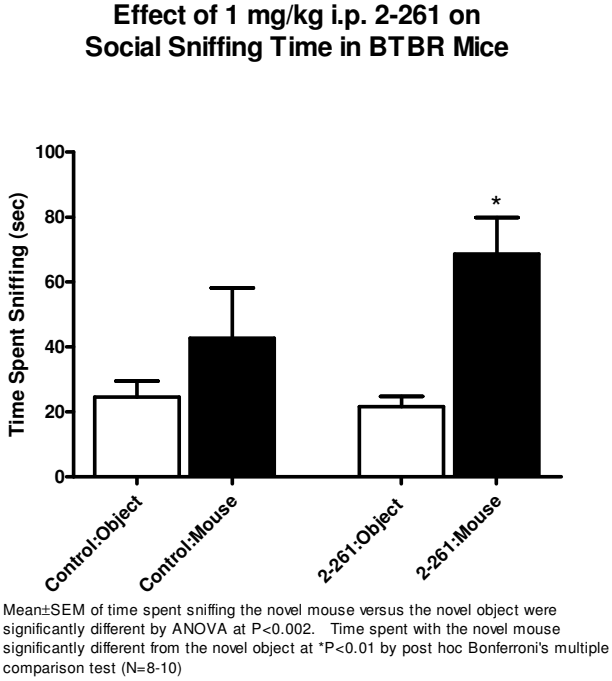

Figure 3: The effect of the 2-261 (1 mg/kg i.p.) on sociability (3 chamber test) in BTBR mice was recorded as the time in seconds (sec) spent sniffing the novel mouse (Mouse) versus the novel object (Object) starting 5 minutes after drug or vehicle administration. The effect of the vehicle (Control) is shown in the left pair of bars and the drug (2-261 treated) effect is depicted in the right pair of bars.

Increasing the dose of 2-261 to 3 mg/kg i.p. resulted in a loss of effect on sociability as shown in figure 4. Although the trend of the drug effect is to increase sniffing times, this effect is undergoing further evaluation to determine if there is a bell-shaped dose response by increasing the dose range of 2-261 that will be tested. Interestingly recent studies with the benzodiazepine agonist clonazepam, a non-selective $GABA_A$ receptor PAM, resulted in a bell-shaped dose response curve in the same strain and behavioral paradigm (Han et al., 2014). Alternatively the variability in the response observed in the 2-261 treated group may also contribute to absence of a statistically significant effect despite a trend toward increased interaction with the novel mouse (figure 4). These dose-dependence studies are underway and will not be complete until August 2014 because of changes in baseline BTBR behavior described below.

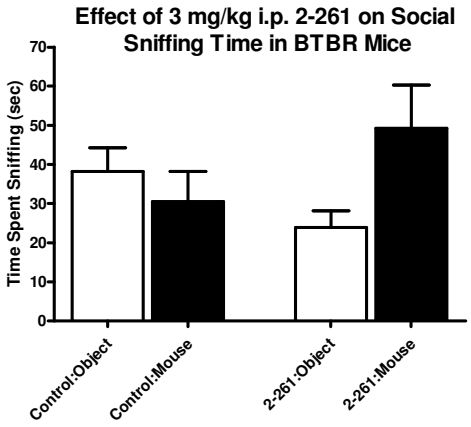

Figure 4: The effect of the 2-261 (3 mg/kg i.p.) on sociability (3 chamber test) in BTBR mice was recorded as the time in seconds (sec) spent sniffing the novel mouse (Mouse) versus the novel object (Object) starting 5 minutes after drug or vehicle administration. The effect of the vehicle (Control) is shown in the left pair of bars and the drug (2-261 treated) effect is depicted in the right pair of bars. No statistically significant differences in times spent sniffing between the various conditions were found after ANOVA.

Studies to determine the effect of α7 nACh receptor PAM AVL-3288 on sociability have been delayed. Mid-way through the dose-dependence studies with 2-261, we noticed that the control behavioral phenotype in the BTBR mice started to drift to the point where they were interacting more with the novel mouse than the novel object. We noticed during the same period that our mice had been moved to a different vivarium where the room was shared with other mice of various strains and the changes in the behavioral phenotype coincided with the move. We have moved the animals back to their original room and the typical behavioral phenotype of the BTBR has been restored. This resulted in a 1 month delay in progress. The extra time was required to determine the reason for the "drift" in the behavioral phenotype of the BTBR mice. This delay was in addition to that related to getting the BTBR breeding colony up to speed.

Task 2h: Sociability – drug effect specificity (C57 strain)

The specificity of the effect of 2-261 at 1 mg/kg i.p. is shown in figure 5 where the drug had no effect on sociability on C57 mice. Thus only the BTBR mice respond to the effect of 2-261 on sociability and demonstrate drug specificity.

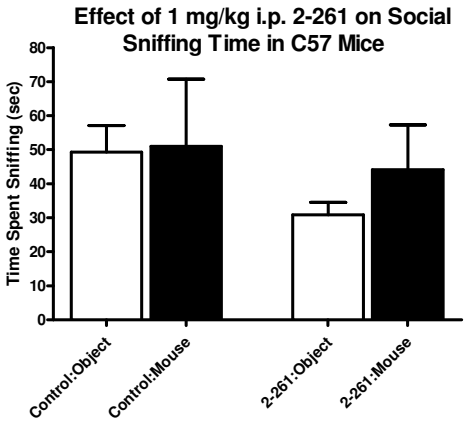

Figure 5: The effect of the 2-261 (1 mg/kg i.p.) on sociability (3 chamber test) in C57 mice was recorded as the time in seconds (sec) spent sniffing the novel mouse (Mouse) versus the novel object (Object) starting 5 minutes after drug or vehicle administration. The effect of the vehicle (Control) is shown in the left pair of bars and the drug (2-261 treated) effect is depicted in the right pair of bars. No statistically significant differences in times spent sniffing between the various conditions were found after ANOVA.

Task 2i: Novel stimulus mice for drug effect specificity

The DBA/2J mice appear to be effective as the novel mice in these social interaction studies where the same DBA/2J mouse is used for every 3 BTBR mice tested for a ratio of 1 DBA/2J to 3 BTBR mice. Initial testing was done with a ratio of 1:1. Subsequent testing with a ratio of 1:3 showed that no differences were observed in any measure when compared to a ratio of 1:1. This observation allowed us to use fewer novel mice per study.

Task 2j & 2k: Self-grooming– phenotype confirmation (BTBR versus C57 strains)

The behavioral phenotype of the BTBR strain of mice was confirmed and consistent with that reported in the literature (Silverman et al., 2012). The phenotype is shown in figure 6 where BTBR mice spend significantly more time self-grooming than the C57 mice. These studies were completed in March, 2014, one month later than anticipated due to the delay in generating sufficient numbers of BTBR mice in the breeding colony.

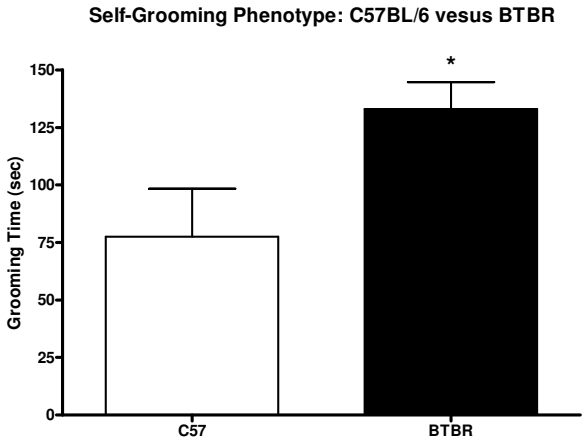

Figure 6: The self-grooming behavioral phenotype of C57 (open bar) versus BTBR (filled bar) strains of mice. Mice were placed in a test chamber and the amount of time (sec) spent self-grooming was recorded during a 5 minute

observation period. A statistically significant difference in grooming time between the strains was observed as determine by an unpaired Student's t-test at *P<0.05.

Task 2l: Self-grooming – drug testing (BTBR strain)

The self-grooming studies with test drugs started in June 2014 with testing of the positive control GRN-529 at 3 mg/kg i.p. (Figure 7). The effect of GRN-529 is similar to that reported in the literature (Silverman et al., 2012). The initial dose of the $GABA_A$ receptor subtype selective PAM 2-261 has been tested and the drug effect is qualitatively similar to that induced by GRN-529 where self-grooming was significantly reduced by drug treatment (Figure 8). The remaining doses of 2-261 and the α7 nACh receptor PAM AVL-3288 will be tested upon confirmation of activity with 2-261. We anticipate that these studies will be completed in October of 2014 because of the delays related to the BTBR breeding colony and change in vivarium environment leading to a behavioral phenotype shift as discussed earlier under task 2g above.

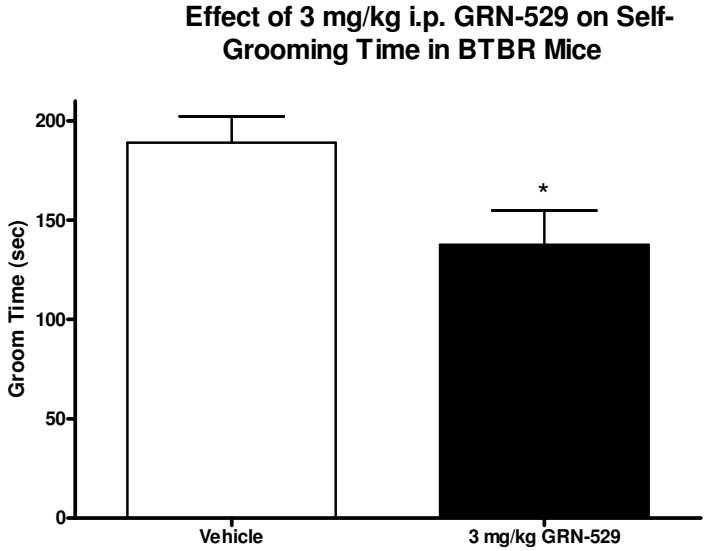

Figure 7: The effect of 3 mg/kg (i.p.) of GRN-529 on self-grooming behavior in BTBR mice. Mice were placed in a test chamber 5 minutes after vehicle (open bar) or drug (filled bar) administration and the amount of time (sec) spent self-grooming (groom time) was recorded during a 10 minute observation period. A statistically significant difference in groom time between the vehicle control and the drug treated groups by an unpaired Student's t-test at *P<0.03 (N=13-17).

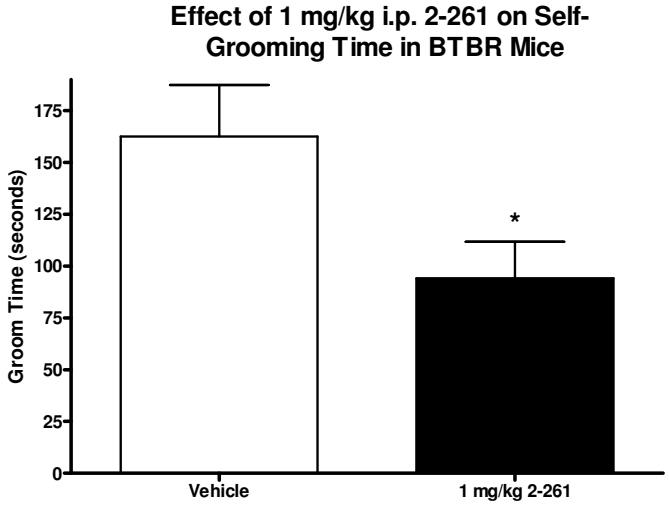

Figure 8: The effect of 1 mg/kg (i.p.) of 2-261 on self-grooming behavior in BTBR mice. Mice were placed in a test chamber 5 minutes after vehicle (open bar) or drug (filled bar) administration and the amount of time (sec) spent self-

grooming (groom time) was recorded during a 10 minute observation period. A statistically significant difference in grooming time between the vehicle control and the drug treated groups by an unpaired Student's t-test at *P<0.05 (N=4-7).

Task 2m: Self-grooming – drug effect specificity (C57 strain)

These studies will commence in early August of 2014 as soon as significant effects are observed in drug testing as specified in task 2l.

4. KEY RESEARCH ACCOMPLISHMENTS:

- *Established BTBR mouse colony and demonstrated behavioral phenotypes similar to certain core symptoms observed in ASD thus providing a useful model of the disease with face validity*

- *Demonstrated that a $GABA_A$ receptor subtype selective PAM, 2-261, ameliorates the deficits of reduced social interaction and increased self-grooming in the BTBR mouse model of ASD thus providing key data in support of the hypothesis that deficits in inhibitory neurotransmission may contribute to these core symptoms of ASD*

- *PAMs which have an obligatory reliance on endogenous levels of neurotransmitter are active in the BTBR model of ASD and thus support the contention that endogenous neurotransmission is sufficient for PAMs to have impact on abnormal behavioral activity in BTBR mice*

5. CONCLUSION:

The first year findings support the hypothesis that the potentiation of inhibitory mechanisms mediated by $GABA_A$ receptor subtypes is a viable strategy to ameliorate two of the core symptoms of ASD, reduced social interaction and repetitive behavior. If these observations translate, it will allow the testing of this concept in clinical populations since non-subtype selective $GABA_A$ receptor PAMs already exist in the form of the clinically used benzodiazepines (BZs). Although the BZs are not ideal drugs because of their side effect profile and abuse potential, they may provide a means to rapidly test the concept clinically if side-effects such as sedation can be mitigated. More importantly our findings set the stage for the continued development of highly selective $GABA_A$ receptor subtype PAMs for the treatment of the core symptoms of ASD. These will be the first drugs purposefully designed to selectively potentiate an inhibitory subsystem in the brain for the treatment of ASD.

In the second year of the grant, we will be completing the assessment of the role of the α7 nAChR in the core symptoms of ASD as observed in the BTBR strain of mice. We will also determine the effect of concurrently modulating the $GABA_A$ and α7 nicotinic receptor systems on learning and memory deficits in BTBR mice. These studies will establish the viability of treating both the core symptoms and learning disabilities by targeting the two systems simultaneously with a single drug.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS: Nothing to report.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: Nothing to report.

9. OTHER ACHIEVEMENTS: Nothing to report.

10. REFERENCES:

Han S, Tai C, Jones CJ, Scheuer T and Catterall WA. Enhancement of inhibitory neurotransmission by $GABA_A$ receptors having α2,3-subunits ameliorates behavioral deficits in a mouse model of autism. Neuron 81:1282-1289, 2014.

Silverman JL, Smith DG, Rizzo SJ, Karras MN, Turner SM, Tolu SS, Bryce DK, Smith DL, Fonseca K, Ring RH and Crawley JN. Negative allosteric modulation of the mGluR5 receptor reduces repetitive behaviors and rescues social deficits in mouse models of autism. *Sci. Transl. Med.* 4(131):131ra51, 2012.

11. APPENDICES: None